Human CD34$^+$ Fetal Liver Stem Cells Differentiate to T Cells in a Mouse Thymic Microenvironment

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Hematopoietic stem cells differentiate in the thymus to T cells along precisely defined intermediates. This process is thymic epithelium dependent and involves cytokines and cell-cell interactions between thymic stroma and T-cell precursors. Here we report that highly purified human CD34^{++} fetal liver stem cells differentiate to mature T cells, when seeded into isolated fetal thymic lobes of severe combined immunodeficient mice, and subsequently cultured in vitro. The human stem cells differentiate sequentially into CD4$^+$CD8$^-$CD3$^-$, CD4$^+$CD8$^+$CD3$^-$, CD4$^+$CD8$^+$CD3$^+$, and finally, CD4$^+$CD8$^-$CD3^{++} and CD4$^-$CD8$^+$CD3^{++} cells. Phenotypic analysis for additional maturation markers showed that these CD4 and CD8 single-positive thymocytes are fully maturate cells. By immunochemistry, human HLA-DR$^+$ cells with a dendritic morphology could be detected. This novel chimeric human-mouse fetal thymus organ culture offers a tool to study human T-cell ontogeny in vitro and is a rapid and reliable test method for T-cell precursor activity of cultured or transfected human stem cells.

HEMATOPOIETIC stem cells are capable of self renewal and differentiation into erythroid, myelomonocytic, and lymphoid lineages.[1,2] Whereas in vitro stem cell assays are available for human myelopoiesis and B-lymphocyte differentiation,[3] this is not the case for T cells. For T-cell development, the three-dimensional structure of the thymic stroma seems to be indispensable.[4] In the thymic stroma, the epithelium is essential for early T-cell differentiation of hematopoietic precursors as well as terminal differentiation to mature T cells.[5,6] The potential of hematopoietic precursor cells to differentiate along the T-cell lineage is therefore difficult to address, because no practical assays exist in which full T-cell differentiation occurs. Recently, severe combined immunodeficient (SCID)-hu mice, chimeras obtained by transplantation of human hematolymphoid tissue into congenitally SCID host have been explored to provide an in vivo culture system for dissecting the human T-cell differentiation.[7-12] CD34$^+$ fetal precursor cells are injected with a microsyringe into HLA-mismatched human thymus fragments, partially depleted of hematopoietic cells by low temperature culture and allowed to develop on engraftment into SCID mice.[8,9] Although this assay requires fetal tissue and is technically cumbersome, it represented a major breakthrough in the study of human hematopoiesis because it allows functional testing of precursor cells and dynamic T-cell differentiation experiments.[8,9] It has been reported recently that this allogeneic organotypic culture system can be adapted to an in vitro system.[13-14] This human fetal thymic organ culture system was used to delineate the early events in human thymic development.[13-14]

Here, we report in vitro human T-cell differentiation of CD34^{++} human fetal liver stem cells in a xenogeneic environment. The experiments are based on the fetal thymus organ culture as described by Kingston et al and Jenkinson et al.[4,15] As thymic environment for human T-cell development, fetal SCID thymus was used. As a result of a defective recombination of the genes coding for the antigen receptor, SCID mice (C.B.−17 *scid/scid*) have an impaired thymocyte maturation.[16] The differentiation of their thymocytes is blocked before the CD4-CD8 double-positive stage,[17] giving space for concurrent maturation of xenogeneic cells. This alternative assay method, developed in our laboratory, eliminates the need of human fetal thymus, the differences in HLA mismatch and in vivo testing.

MATERIALS AND METHODS

Preparation of human CD34^{++} fetal liver cells. Human fetal liver tissues were obtained after legal termination of pregnancy, and obtained and used following the guidelines of the Medical Ethical Commission of the University Hospital of Ghent. Human fetal liver cells were isolated by gentle disruption of the tissue in complete medium (Iscove's modified Dulbecco's medium/10% fetal calf serum [FCS]; GIBCO, Paisley, Scotland), followed by density gradient centrifugation over Lymphoprep (Nyegaard, Oslo, Norway). Cells were washed and resuspended in 90% FCS/10% dimethyl sulfoxide and frozen in liquid N_2. After thawing, fetal liver cells were washed and immunofluorescently labelled with CD3 (Leu-4 fluorescein isothiocyanate [FITC]), CD4 (Leu-3a FITC), CD8 (Leu-2a FITC), CD19 (Leu-12 FITC), CD1 (CD1a FITC; Serotec, Oxford, United Kingdom), and with CD34 (HPCA-2 PE) (unless specified, all MoAbs were from Becton Dickinson Immunocytometry Systems, Mountain View, CA). CD34^{++} CD4$^-$CD8$^-$CD3$^-$CD19$^-$CD1$^-$ cells were sorted on a fluorescence-activated cell sorter (FACS) vantage (Becton Dickinson). The sorted cells were transferred to murine thymic lobes by the hanging drop method.[15]

Mice. Fourteen to 15 day pregnant C.B.-17 SCID mice were obtained from our own specific pathogen-free breeding facility.

Fetal thymus organ culture (FTOC). Thymic lobes were prepared from fetal day 14 to 15 SCID mice. Hanging drops were prepared in Terasaki plates by adding in each well 25 μL of complete medium containing 10,000 or less sorted cells to one thymic lobe. The plates were immediately inverted to form hanging drops and incubated during 48 hours in a humidified incubator (7.5% CO_2 in air, 37°C). After incubation, the lobes were removed from the hanging drop, washed, and put on the surface of a nuclepore filter in organ culture in an incubator. At different time points, thymocytes were recovered by mechanical disruption of the thymic lobes with a small tissue grinder. Thymocytes were stained with eosin and

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counted with a hematocytometer. At least 80% of the cells were viable.

Flow cytometry. Before labeling, cells were suspended in phosphate-buffered saline (PBS) 1% bovine serum albumin (BSA) −0.1% NaN_3 and the Fc receptor of the mouse thymocytes was blocked by preincubation for 15 minutes with anti-FcγRII/III MoAb (clone 2.4.G2)[18] to avoid aspecific binding of antibodies by the murine thymocytes. Subsequently, the cells were stained with a panel of MoAbs, as indicated. The MoAbs used were rat anti-mouse CD45 (CD45 R-PE, MI-9.3 HL; Gibco BRL: Immunoselect or CD45-Biotin, 30F11.1; Pharmingen, San Diego, CA), CD1a (B-B5; Serotec, Oxford, United Kingdom) and the following mouse anti-human MoAbs from Becton Dickinson, CD3 (Leu-4 FITC, PE, or biotin), CD4 (Leu-3a FITC or PE), CD8 (Leu2a FITC or biotin), CD14 (Leu-M3 PE), CD19 (Leu-12 PE), CD34 (HPCA-2 FITC or PE), CD45 (HLe-1 FITC), anti–T-cell receptor (TCR)–$\alpha\beta$ (TCR-1 FITC), anti-TCR-$\gamma\delta$ (anti-TCR-$\gamma\delta$/1 FITC). The biotinylated antibodies were revealed with second-step streptavidin cychrome (Pharmingen). All MoAbs against human antigens were checked for negative staining on SCID thymocytes after blocking with 2.4G2 MoAb. Isotype controls were also included in most staining series and were found to be negative. The cells were analyzed on a FACScan (Becton Dickinson) with an argon-ion laser tuned at 488 nm. Forward light scattering, orthogonal scattering, and three fluorescence signals were determined on 10,000 cells and stored in listmode data files. Data acquisition and analysis was performed with Lysis 2.0 software (Becton Dickinson). Dead cells were gated out by propidium iodide exclusion (PI). In most cases, viable human cells were gated by exclusion of mouse CD45-PE or mouse CD45-biotin-streptavidin-cychrome–positive cells and PI-positive cells.

Immunocytochemistry. After organ culture, thymic lobes were frozen in liquid nitrogen after embedding in OCT compound (Miles Scientific, Naperville, IL). Immunochemistry was performed on 5 to 6 μm-thick cryostat sections fixed in cold (4°C) acetone. Endogenous peroxidase was destroyed (PBS + 3% H_2O_2, 10 minutes) and nonspecific antibody binding was blocked throughout the procedure using 3% BSA in PBS (Calbiochem, La Jolla, CA) and by preincubation in 3% of normal rabbit Igs (Dako, Prosan, Gent, Belgium) for 30 minutes.

Sections were incubated sequentially, with intervening washes, in anti-human HLA-DR (Becton Dickinson) or in anti-human leucocyte-common antigen (L-CA, Dako), which recognizes the CD45 protein found on all cells of hematopoietic origin except erythrocytes,[19] followed by peroxidase-conjugated anti-mouse Igs (Dako). Sections were developed with 3,3′-diaminobenzidine as substrate (UCB, VEL Leuven, Belgium). Controls were incubation with the secondary antibody only, or incubation with unrelated mouse Igs instead of the specific antibodies.

RESULTS AND DISCUSSION

As shown in Fig 1A, $CD34^{++}CD4^{-}CD8^{-}CD3^{-}CD1a^{-}CD19^{-}$ hematopoietic stem cells were highly enriched from human fetal liver (gestational age ranged from 18 to 22 weeks) by flow cytometric sorting. These highly purified $CD34^{++}$ cells (>99.7%) were seeded into single thymic

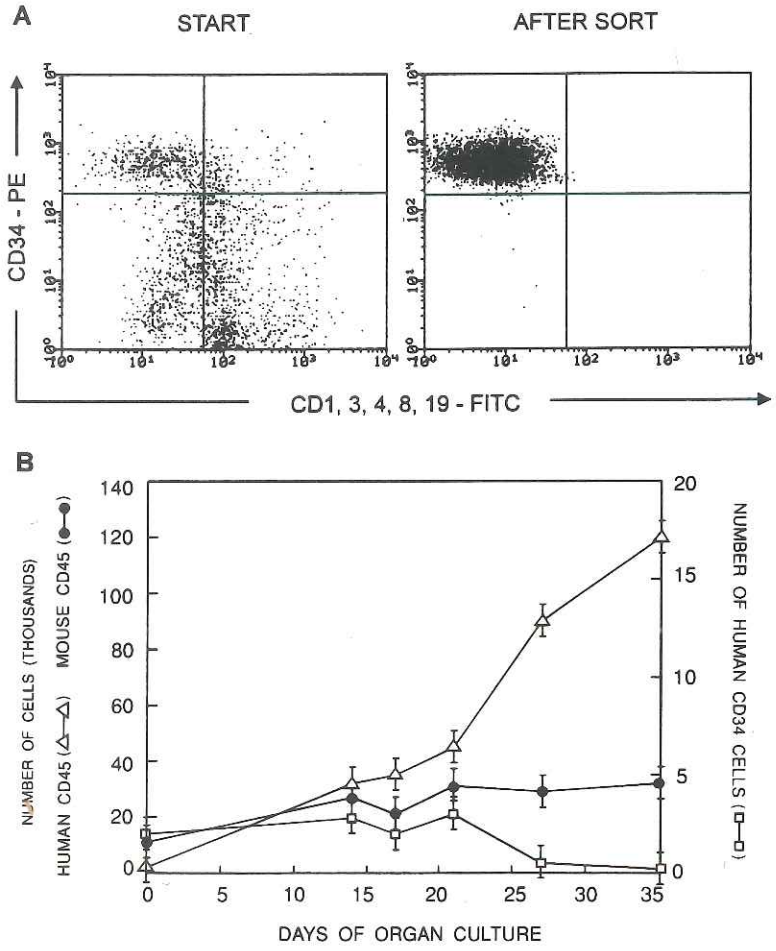

Fig 1. Growth kinetics of human and murine thymocytes in chimeric organ cultures. (A) Flow cytometric analysis of human fetal liver cells stained with CD34-PE and FITC-labelled CD1, CD4, CD8, CD3, and CD19 before and after sorting. The cells were purified by gating on $CD34^{++}$ and $CD4^{-}$, $CD8^{-}$, $CD3^{-}$, $CD1^{-}$, $CD19^{-}$. (B) Growth kinetics of human and murine thymocytes. 10,000 human $CD34^{++}$ fetal liver cells were seeded in SCID murine fetal thymi by hanging drop. After 48 hours (day = 0) unseeded cells were washed away and the lobes were cultured on Nuclepore filters in FTOC. At different time points, cells were counted and stained with human CD45 and mouse CD45 or with CD34 at the indicated times and assessed by flow cytometry (FACScan). The cell number for each subset was calculated by multiplying the total cell number with the percentage of cells that stained for a given antigen divided by 100. Data are expressed as mean values (±1 SD) of 3 to 4 independent experiments, each of which contained at least three lobes per data point. The scale on the left axis is for human CD45 and mouse CD45 cells, the smaller scale on the right axis is for CD34 cells. A gradual increase in human cells, a constant level of mouse cells and a constant level of $CD34^{+}$ until day 21 followed by a decline. Data are representative for three experiments.

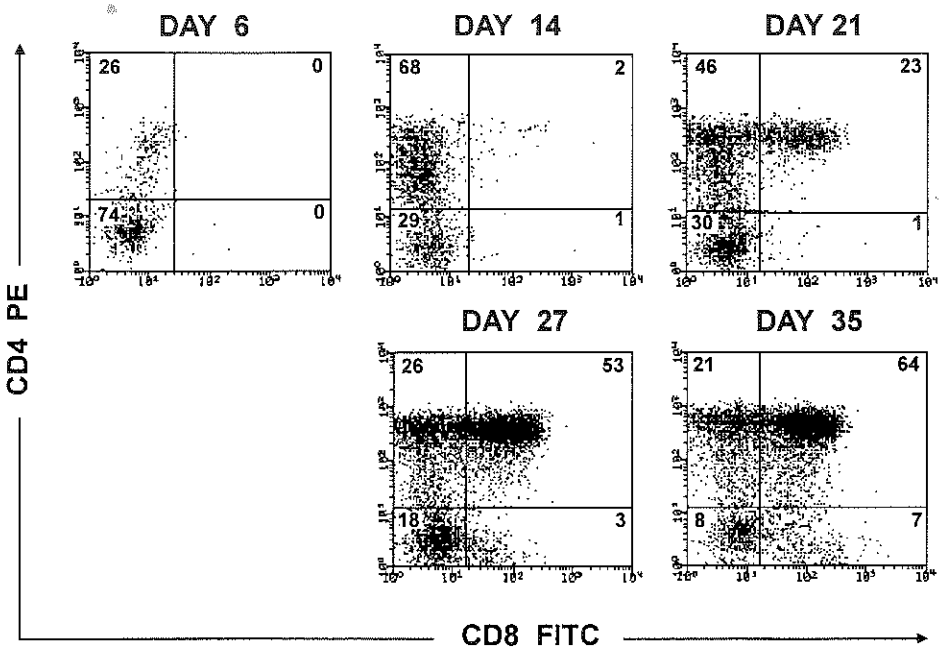

Fig 2. Kinetic analysis of the expression of CD4, CD8. Human-mouse chimeric cultures were set up as described in Fig 1. Thymocytes were recovered, counted, and stained for coordinate expression of CD4 and CD8 at the indicated times. Data are representative for two to four experiments (with at least 4 lobes each) per timepoint.

lobes of fetal day 14 to 15 SCID mice. When 10,000 cells were placed in a hanging drop, together with one thymic lobe, approximately 2,000 cells entered the lobe during a time period of 48 hours (day = 0). As shown in Fig 1B, the murine thymus supported the growth of human thymocytes as the number of human $CD45^+$ cells increased progressively. The number of endogenous SCID thymocytes remained at low levels. Starting from approximately 2,000 human cells in a lobe, about 120,000 human cells were obtained after 5 weeks of culture, which represents a 60-fold increase. The absolute number of $CD34^+$ cells remained constant during the first 3 weeks of culture, and declined thereafter (Fig 1B), suggesting that the thymic microenvironment cannot maintain self-renewal of the hematopoietic stem cells. The pre–T-cell precursor frequency in the sorted $CD34^{++}CD3^-CD4^-CD8^-$ cell population was determined by limiting dilution using automatic cell deposition unit (Becton Dickinson). Groups of 20 lobes were used for transfer of 1, 10, or 100 cells/lobe by hanging drop. The proportion of repopulated lobes containing human $CD45^+CD4^+CD8^+$ cells in each group was determined by FACS analysis. After 14 to 21 days, 0/20 lobes were repopu-

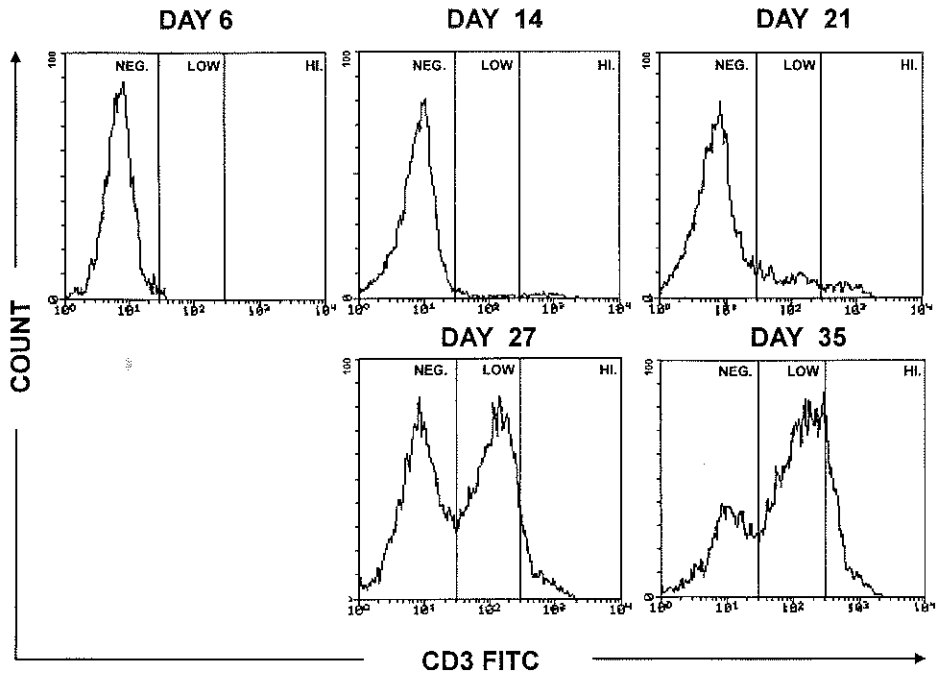

Fig 3. Kinetic analysis of the expression of CD3. Human-mouse chimeric cultures were set up as described in Fig 1. Thymocytes were recovered, counted, and stained for expression of CD3 at the indicated times. Data are representative for two to four experiments (with at least 4 lobes each) per timepoint. Data are presented as CD3 histograms at different time points.

lated in the group in the group of 1 cell/lobe, 1/20 lobes in the group of 10 cells/lobe and 15/20 in the group of 100 cells/lobe (data not shown). This shows that the chimeric human-mouse organ culture is an assay system for T-cell precursors at the clonal level. When human differentiation markers were studied, the successive appearance of different subsets was noted. Analysis of the cells that seeded the thymic lobe (day 0) showed that at least 80% of the cells were still $CD34^{++}$. After 6 days of FTOC, a small population (~20%) expressed low levels of CD4 (Fig 2). These $CD4^{low}$ cells precede the emergence of $CD4^{high}$ cells. After 14 days of culture, $CD4^{low}CD3^-$ and $CD4^{high}CD3^-$ cells were present. These cells were $CD1a^+$ and $CD34^-$ (data not shown). At that time, only a small number of cells expressed CD3 (Fig 3). In the human thymus, this $CD1^+CD4^+CD8^-CD3^-$ subset has been reported to contain the highest proportion of cycling cells[20] and it was suggested that this subset represents an early transitional stage between $CD4^-CD8^-$ and $CD4^+CD8^+$ thymocytes.[12,13,14,20,21] Besides $CD4^+$ cells, a small number of cells expressed CD3 at high levels. Virtually all these cells expressed the $\gamma\delta$ T-cell receptor (data not shown), indicating much faster differentiation kinetics of $\gamma\delta$ T cells compared with $\alpha\beta$ T cells. After 21 days, $CD4^+CD8^+$ cells were present, part of them were $CD3^+TCR\alpha\beta^{low}$ cells. Virtually all cells were $CD1a^+$ (data not shown). After 27 days, single $CD4^+$ and $CD8^+$ thymocytes appeared with high CD3 expression. By 35 days of culture, a phenotypically normal thymus was seen: 64% of the population were $CD4^+CD8^+$ cells, 21% were $CD4^+CD8^-$ cells, and 7% were $CD8^+CD4^-$ cells. $CD4^-CD8^-$ cells made up about 8% of the total population (Fig 2) and did not contain $CD34^+$ cells, whereas virtually all cells in this subset expressed the TCR, half of them were TCR-$\alpha\beta$ and half of them were TCR-$\gamma\delta$ cells. Half of the $CD4^+$ cell population had characteristics of immature cells as they were $CD3^-$ and $CD1^+$ (data not shown). To show terminal differentiation of part of the CD4 and CD8 single positive cells, the cells were analyzed for additional maturation markers (Fig 4): single positive CD4 and CD8 cells (data not shown) were present with high expression of TCR-$\alpha\beta$. These cells had a $CD1^-$, HLA class I^{++} phenotype that is compatible with terminally maturated thymocytes (Fig 4). In a typical experiment, after 35 days of culture, a TCR-$\alpha\beta$ $CD3^{hi}$ cell population was obtained with a staining intensity of CD3 comparable to peripheral T cells. These "mature" thymocytes displayed 6% of $CD4^-$ $CD8^+$ cells and 6.5% of $CD4^+CD8^-$ cells of the total thymocytes (data not shown). In contrast to normal T-cell development of TCR-$\alpha\beta$ and TCR-$\gamma\delta$ cells, no CD19 nor CD14 positive cells were found by FACS analysis, suggesting that B lymphopoiesis and monocytopoiesis does not occur. Neither were $CD56^+CD3^-$ natural killer cells found. These populations are normally present as a minor fraction in human thymus.[22] With immunochemistry, human cells displaying a dendritic morphology were observed. On sections of fetal thymic lobes cultured with human fetal stem cells for 9 days, the cortex and the medulla were morphologically indistinguishable (Fig 5A). At this stage, anti-human HLA-DR and L-CA (CD45) antibodies reacted with a small number of cells: some of the cells stained by anti-human HLA-DR displayed dendritic morphology, with long cytoplasmic processes (Fig 5C).

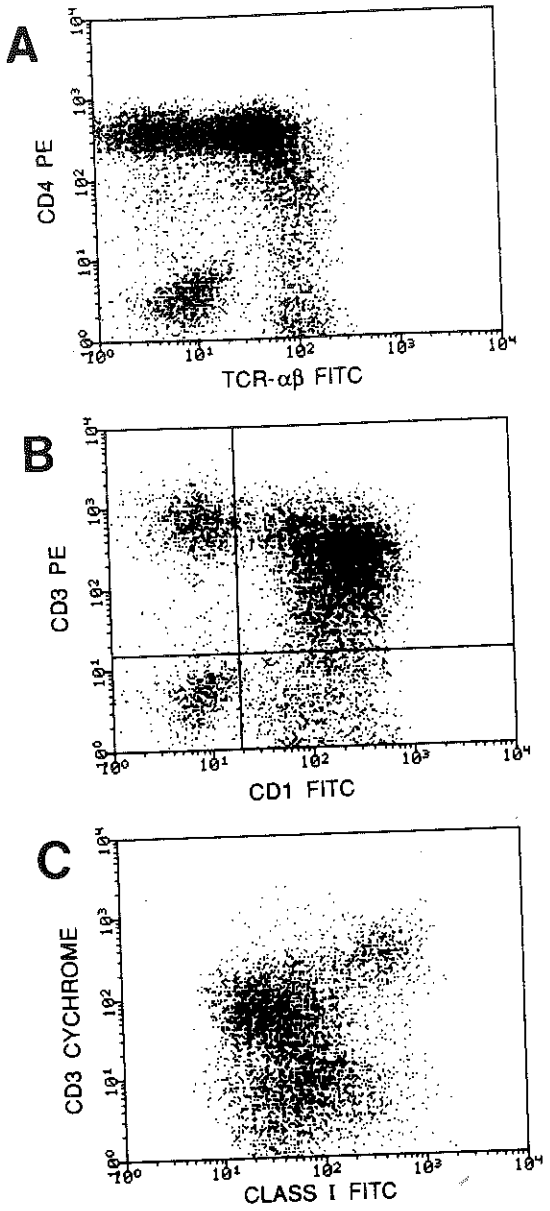

Fig 4. Phenotype of human thymocytes after 5 weeks of organ culture. TCR-$\alpha\beta$/CD4, CD3/CD1, CD3/HLA-class I dot plots, with the presence of $CD4^+$ TCR-$\alpha\beta^{hi+}$ (A), $CD3^+CD1^-$ cells (upper left quadrant) (B) and $CD3^+$ with high HLA-class I^{hi+} expression (C). These phenotypes are seen in differentiated and positively selected thymocytes.

Later (day 35), the morphology of thymic lobes is similar to that observed in a normal thymus with a high density of thymocytes within the cortex and stromal cells easily seen in the medulla. At this stage, anti-human CD45 antibodies reacted strongly with the majority of cortical thymocytes, indicating their human origin, whereas only scarce lymphocytes are labeled in the medullary area (Fig 5C). Anti-human HLA-DR reacts with cells displaying a morphology of dendritic cells: some of these HLA-DR–positive cells are located in the cortex, the majority of them being observed at the cortico-medullary junction (Fig 5D).

In conclusion, we show that human fetal liver stem cells

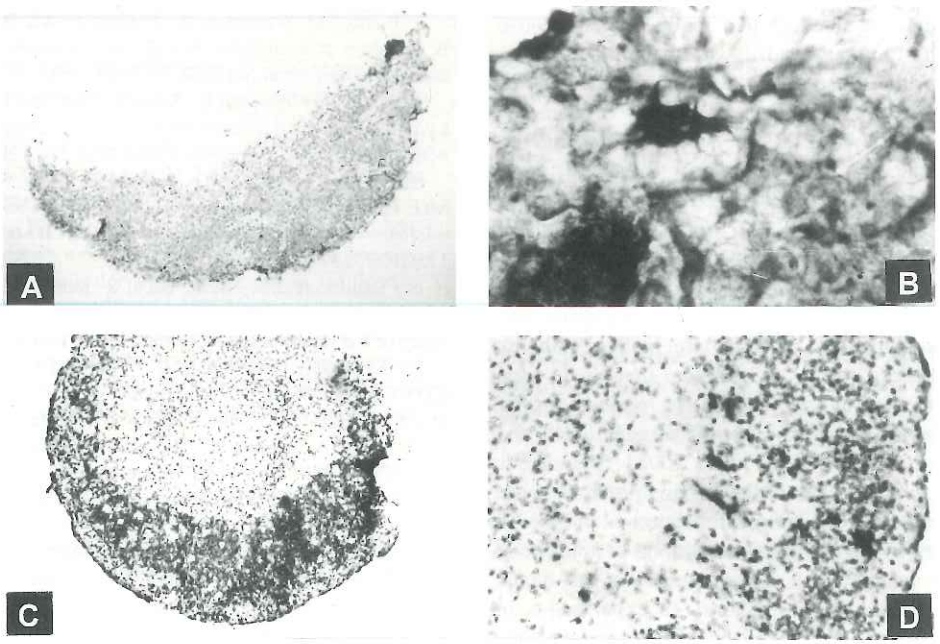

Fig 5. Immunohistochemical analysis of thymic lobes. Frozen sections of thymic lobes after 9 days (A and B) and 35 days of culture (B and D) were stained with the MoAb CLA (anti-human CD45: A and C) or anti–HLA-DR (B and D). Original magnification × 10 (A and C); × 40 (D); × 100 (B).

differentiate normally to TCR-$\alpha\beta$ and TCR-$\gamma\delta$ cells in a murine SCID fetal thymic environment. For the first time, we show that highly purified $CD34^{++}$ fetal liver cells under stringent conditions of purity (99.7% pure) and at very low cell numbers (100 cells:lobe) are able to differentiate to the complete T-cell lineage in a mouse thymic microenvironment. Previously, it was shown that unpurified thymocytes are able to survive and possibly to differentiate in xenogeneic microenvironment, but this depended on the introduction of stromal elements of human origin.[23,24] We reported that semipurified fetal liver stem cells, obtained by depletion of mature CD3, CD4, and CD8 cells by immunomagnetic bead depletion, were also able to differentiate in a xenogeneic FTOC.[25] Here, we show that highly purified $CD34^{++}$ precursor cells depleted of stromal elements differentiate to mature T cells in murine fetal thymic rudiments. The suggestion that species-specific signals derived from either epithelial or mesenchymal origin is required for T-cell development[23,24] is challenged by our data. In the murine system, the development of $CD3^-CD4^-CD8^-$ T-cell precursors into double-positive cells requires both the support of epithelial and mesenchymal cells, whereas later stages of maturation can be supported by epithelial cells alone.[26] Although we cannot formally disprove that human epithelial cells derive from $CD34^{++}$ stromal progenitors in the fetal liver specimen, it is likely that in our model the epithelial cells are from murine origin. Our experiments show that the interactions between epithelial stromal cells and hematopoietic precursors that are necessary for the transition of $CD4^-CD8^-$ to $CD4^+CD8^+$ are not species-specific, whereas the mesenchymal support can be derived from the $CD34^{++}$ cells, in view of recent data that these cells also have the potentiality to differentiate in stromal cells.[27] Positive selection of thymocytes is directed by ligation of cell surface receptors.[28-30] The ability of interaction of human CD8 with the $\alpha 3$ domain of murine MHC class I[31] and of human CD4 with murine MHC class II molecules[32-34] may partly explain how mouse epithelial cells can support human thymopoiesis. However, because we have found that human $HLA\text{-}DR^+$ cells with dendritic morphology are present early in the chimeric FTOC, we have to consider that human dendritic cells can induce thymocyte selection. The presence of cells with dendritic morphology of human origin after transfer of purified $CD34^{++}$ cells is in line with recent data showing that in mouse, thymic dendritic cells and T cells develop simultaneously in the thymus from a common a precursor[35] and that human $CD34^+$ precursor cells can differentiate not only to hematopoietic cells but also to bone marrow–derived thymic stromal cells.[27] Therefore, another possibility is that in our cultures, interactions between human T cells and dendritic cells direct T-cell differentiation from cortical-type double-positive thymocytes toward medullary-type single-positive thymocytes. It has indeed been shown that in the murine system bone marrow–derived stromal cells also are sufficient to direct positive selection of $CD8^+$ T cells, although at a reduced rate compared with positive selection by thymic epithelial cells.[36]

Yeoman et al[37] have shown that purified $CD34^+$ cells from umbilical cord blood or adult bone marrow are able to produce $CD4^+CD8^+$, and CD4 and CD8 single-positive cells in mouse FTOC. Under these conditions, mature cells were already obtained after 1 week of FTOC, $CD4^+CD8^+CD3^-$ cells, which in normal thymic differentiation preceeds single positives were absent, and the colonization depended on the presence of $CD7^+$ progenitor T cells.[37] The slower differentiation kinetics obtained in our experiments is in agreement with that reported for mouse fetal liver stem cells.[6,38] The

slower kinetics may also reflect that in the human-mouse FTOC the growth of human dendritic/stromal cells precedes and is required for the subsequent development of human T cells. In our cultures, we find $CD4^+CD8^+CD3^-$ cells and an emergence of populations of thymocytes in a time-ordered sequence in accordance with normal human thymic differentiation. Finally, the purified $CD34^{++}$ cell population used in our experiments contained no CD7 bright cells, but about 20% of cells stained weakly for CD7. In a series of experiments, where the starting $CD34^{++}$ population was supplementarily depleted for CD7, we observed a similar differentiation (data not shown). Our findings are in line with the observations of Barcena et al,[14] who found that $CD7^-$ fetal liver cells can differentiate to T cells. It requires further investigation to determine whether the difference in reconstitution kinetics, presence of $CD3^-CD4^+CD8^+$ thymocytes and dependence of $CD7^+$ cells in the starting population are because of differences in the stem cell properties of embryonic fetal liver versus the umbilical cord blood or adult bone marrow. Another factor that is of importance in our hands is the purity of the starting population. In experiments in which cell populations are contaminated with low amounts of mature T cells, these contaminants expand fast and to high numbers and interfere with the T-cell differentiation pattern (data not shown). Finally, we showed that fetal thymus organ culture is a reproducible method to support the full range of human T-cell maturation, as six independent sources of fetal liver cells and 99% of the cultures (600/608 lobes examined) were successful. This method is a unique tool to study early steps of T-cell differentiation, and to explore the role of cytokines and ligands in this process. In addition, because stem cells are targets for gene therapy, the stability of gene expression can be followed throughout the T-cell differentiation process. Because the thymus is susceptible for human immunodeficiency virus (HIV) infection, this method should prove useful to evaluate if gene therapy strategies are effective in the cure of HIV infection.

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